Isolation and total synthesis of kirkamide, an aminocyclitol from an obligate leaf nodule symbiont

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Isolation and Total Synthesis of Kirkamide, an Aminocyclitol from an Obligate Leaf Nodule Symbiosis

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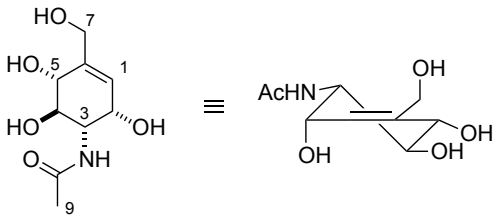
Abstract: The new C_7N aminocyclitol kirkamide (**1**) was isolated from leaf nodules of the plant *Psychotria kirkii* using a genome driven-^{1}H-NMR guided-fractionation approach. The structure and absolute configuration were elucidated by HRMS, NMR and single crystal X-ray structure analysis. An enantioselective total synthesis was developed, which delivered kirkamide (**1**) on a gram scale in 11 steps, featuring a Ferrier carbocyclization and a Pd-mediated hydroxymethylation. We propose that kirkamide is synthesized by *Candidatus* Burkholderia kirkii, the obligate leaf symbiont of *Psychotria kirkii*. Kirkamide (**1**) was shown to be toxic to arthropods and insects, suggesting a protective role of bacterial secondary metabolites in the *Psychotria/Burkholderia* leaf nodule symbiosis.

Over the last years, investigations of the molecular interactions of symbiotic bacteria with their hosts have transformed a number of research fields, from the human microbiome to ecology to crop protection, and these studies have revealed chemical aspects for successful cooperation on a molecular level.[1,2] However, one of the main challenges in this scientific approach resides in the difficulties associated with the cultivation of the bacterial symbionts in the absence of their hosts, which hampers chemical analyses due to the limited amount of material.[3] Therefore, many studies have focused on systems where material supply, both for genetic and chemical studies, can be secured. As a result, many symbioses between animals and prokaryotes have been investigated.[1-3]

In stark contrast to animals, only one symbiosis has been described in higher plants in which the bacteria are vertically transmitted and which can be considered obligate. Bacterial leaf nodule symbiosis has been first described in 1902, and studies over the last century have provided ample evidence that leaf nodules represent the arguably most complex, and certainly most intimate association between bacteria and higher plants, as survival of both partners is dependent on the successful establishment of a symbiosis.[4] In spite of over 100 years of research, the chemical constituents involved in symbiosis have remained elusive. In this study, we present the structure elucidation, total synthesis, and biological evaluation of a new natural product, kirkamide (**1**), from the bacterial leaf symbiont of *Psychotria kirkii* (*P. kirkii*), and discuss the potential role of this C_7N aminocyclitol in the symbiosis.

Analysis of the genome of the uncultured *Candidatus* Burkholderia kirkii (*B. kirkii*), identified genes potentially involved in the biosynthesis of a putative C_7N aminocyclitol.[5,6] These genes have no other known homologues within the genus *Burkholderia* and have been maintained in the genome of *B. kirkii* despite rampant genome erosion. Moreover, the C_7N aminocyclitol family of compounds is known to display a wide range of biological activities.[7] Together, these facts point towards a key role of these secondary metabolite(s) in leaf nodule symbiosis. We therefore decided to isolate and characterize this putative new C_7N aminocyclitol synthesized by the endosymbiont of *P. kirkii*.



Kirkamide (**1**)

As aminocyclitols display the resonance of a characteristic methylene proton around 6 ppm in the ^{1}H-NMR spectrum, we opted for an unusual, genome-based, NMR-guided fractionation approach. In order to overcome sample limitation, we used a 1.7 mm micro-cryoprobe 600 MHz NMR spectrometer in the early stage of the discovery process. Submicrogram amounts of sample were sufficient to record a ^{1}H-NMR spectrum that confirmed the presence of a C_7N aminocyclitol in the extract. Importantly, this signature could not be detected in crude extracts from stunted, aposymbiotic *P. kirkii* plants. After multiple RP-HPLC runs on a Synergi Hydro column, a mixed fraction with the appropriate ^{1}H-NMR signal was obtained. Sucrose was identified as the contaminant, which was removed by treating the crude extract under acidic conditions. The final, successful isolation of the new C_7N aminocyclitol kirkamide (**1**) was then possible by a combination of RP-HPLC and Cu(II) coated preparative thin layer chromatography, a procedure reported for the separation of saccharides.[8]

The high resolution ESI-MS of kirkamide (**1**) displayed an exact mass of *m/z* 240.0844, which supports the molecular formula $C_9H_{15}NO_5Na$ for the $[M + Na]^+$ pseudomolecular ion. ^{1}H and ^{13}C NMR spectroscopic data (DMSO-d_6) of kirkamide (**1**) were compared with those reported for streptol,[9] which suggested a different substitution pattern on C-2, as well as the presence of an acetyl group. This group was established as an NH-acetyl fragment based on HMBC correlations between the quaternary

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carbonyl C-8 with the NH and H-9 proton signals. The assignment of the carbocyclic core structure was then deduced using the ^{1}H-^{13}C HMBC correlations between H-5, H-7a and H-7b, and C-6 as well as H-5 and C-1 and ^{1}H-^{1}H COSY correlations between H-1 and H-2, H-2 and H-3, H-3 and H-4, NH and H-3, H-4 and H-5 and H-5 and H-1. The relative configuration was then assigned comparing the *J*-coupling constants between H-2 and H-3, H-3 and H-4, and H-4 and H-5 in DMSO-d_6 (Table 1) with the one reported for streptol (D_2O).[9] To exclude the influence of the solvent, the recorded spectroscopic data of valienamine in DMSO-d_6 (see Supporting Information) showed that the compounds shared the same conformation with H-2 in equatorial position and H-3, H-4 and H-5 in axial position. Finally, X-ray crystal structure analysis (Figure 1) established the constitution and configuration of kirkamide (**1**).

Table 1. NMR Spectroscopic data (500 MHz, DMSO-d_6) of kirkamide (**1**)

C/N no.	δc, type	δ_H (*J* in Hz)	HMBC[a]
1	121.5, CH	5.63, dq[b] (4.8, 1.5)	3, 5, 7
2	64.3, CH	3.99, m	
3	53.1, CH	3.63, ddd (10.9, 8.1, 3.9)	
NH		7.36, d (8.1)	8
4	70.4, CH	3.56, dd (10.9, 7.1)	3, 5
5	72.9, CH	3.79, d (7.1)	1, 4, 6
6	142.5, C		
7a	60.8, CH_2	4.01, d (14.8)	1, 6
7b		3.95 d (14.8)	1, 6
8	169.2, C		
9	23.0, CH_3	1.85, s	8

[a] HMBC correlations are given from proton(s) stated to the indicated carbon atom. [b] Apparent splitting pattern.

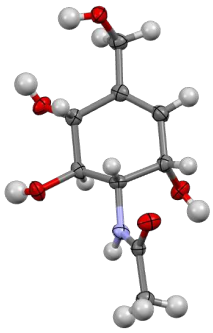
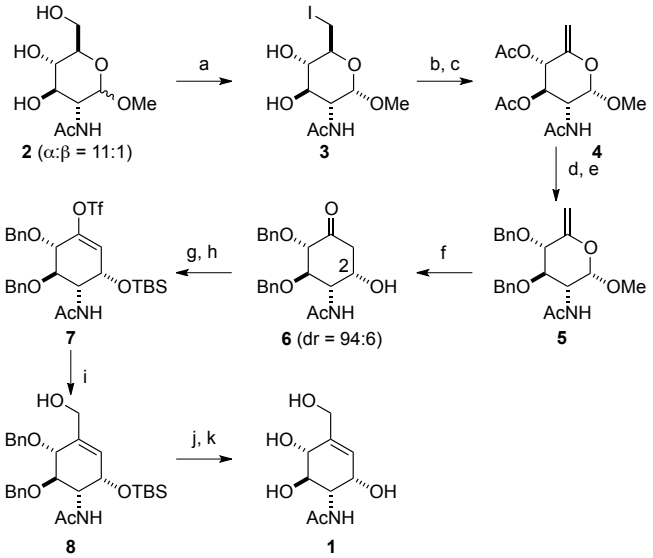


Figure 1. Single crystal X-ray structure analysis of kirkamide (**1**). Cambridge Crystallographic Data Centre accession number: CCDC 1054238.

After the structure of kirkamide (**1**) was established, we turned to total synthesis in order to obtain large quantities of this compound for subsequent biological studies. The synthesis started with the known methyl-*N*-acetyl-D-glucosamine **2**,[10] which was transformed via a Garegg-Samuelsson reaction using PPh_3, imidazole and I_2 to the iodide **3**.[11] A one step procedure to reach the benzyl protected enol ether **5** was evaluated by reacting the substrate **3** with NaH and BnBr, unfortunately a bicyclic by-product was observed as the major compound. Therefore, the iodide **3** was first acetylated followed by AgF-mediated elimination to give the desired enol ether **4** in 50% yield over 3 steps.[12] The protecting groups were exchanged from acetyl to benzyl in two steps affording the key intermediate **5**.[12] The conversion of the exocyclic enol ether to the cyclohexanone **6** was achieved via a Ferrier carbocyclization utilizing $HgSO_4$ as a catalyst under microwave conditions.[13,14] To our delight, excellent diastereoselectivity (94:6) at C-2 in cyclohexane **6** was obtained in the course of this reaction. Protection of this β-hydroxy ketone appeared daunting at first, as elimination of H_2O seemed plausible, but silyl protection and triflation using the Comins' reagent[15,16] proceeded smoothly to give the Stille coupling precursor **7**. The cross-coupling reaction[17] provided the primary alcohol **8**, and subsequent deprotection by TBAF and Birch conditions[18] resulted in synthetic kirkamide (**1**). All the synthetic steps up to the final deprotection were amenable to gram scale preparation, which demonstrates the robustness of this route. The spectroscopic data of synthetic kirkamide (**1**) confirmed the structure of the natural product and finally allowed for the assignment of all OH resonances in the ^{1}H-NMR spectrum (Supporting Information).



Scheme 1. Synthesis of kirkamide (**1**). Reaction conditions: a) Ph_3P, Imid, I_2, THF, reflux, 15 min; b) Ac_2O, pyridine, RT, 24 h; c) AgF, pyridine, RT, 48 h, exclusion of light, 50% over 3 steps; d) $NH_{3(gas)}$, MeOH, RT, 3 h; e) NaH, BnBr, DMF, 0°C, 12 h, 36% over 2 steps; f) $HgSO_4$ dioxane : aq. H_2SO_4 (5 mM) 2:1, μw, 60°C, 15 min; g) TBSOTf, 2,6-lutidine, THF, 0°C, 12 h, 63% over 2 steps; h) Comins' reagent, NaHMDS, THF, −78°C, 5 min, 62%; i) Bu_3SnCH_2OH, $Pd(PPh_3)_4$, LiCl, dioxane, μw, 105°C, 1 h, 85%; j) TBAF, THF, RT, 3 h, 98%; k) $Na/NH_{3(liq)}$, THF, −78°C, 30 min, 59%. Imid: imidazole, THF: tetrahydrofuran, DMF: dimethylformamide, TBS: *tert*-butyldimethylsilyl, HMDS: hexamethyldisilazane. TBAF: tetra-*n*-butylammonium fluoride

We tested cytotoxic activity of pure synthetic kirkamide (**1**) using a brine shrimp lethality assay on instar II nauplii of *Artemia*.[19] Synthetic kirkamide (**1**) displayed an LC_{50} value at 48 h of 0.84 μg/mL, indicating that it is toxic to crustaceans. Since insects play a major role as herbivores and bacterial secondary metabolites could play a protective role for the plant, we also

tested insecticidal activity of synthetic kirkamide (**1**) to wild-collected pollen beetles (*Meligethes aeneus*). In our assay, pollen beetles fed with pollen tainted with 0.3% w/w of kirkamide (**1**) reached mortality levels of up to 90% at 14 dpi.

In summary, kirkamide (**1**), a new natural product possessing a C_7N aminocyclitol core structure was isolated from leaf nodules of *Psychotria kirkii*. Genomic evidence strongly suggests that the *B. kirkii* symbiotic bacteria are responsible for the synthesis of kirkamide.[6] To investigate the biological activity of kirkamide (**1**), we developed a gram scale total synthesis using a Garegg-Samuelsson reaction as a key step to efficiently provide the enolether intermediate **5**. By this approach, kirkamide (**1**) was synthesized via a Ferrier carbocylization and a Stille cross-coupling. Kirkamide (**1**) is toxic to arthropods and insects, suggesting a protective role of bacterial secondary metabolites in the *Psychotria/Burkholderia* leaf nodule symbiosis.

Keywords: natural products • organic synthesis • plants • bacteria • symbiosis

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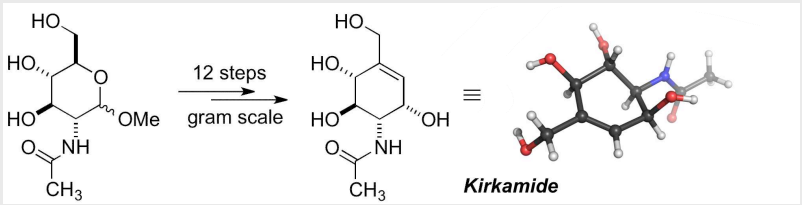

Made for sharing: The chemical nature of the interaction between the plant *Psychotria kirkii* and its bacterial symbiont remains unclear. The genomic analysis of the microorganism, forming nodules under the leaves of the host, suggested the presence of a C_7N aminocyclitol. We report the isolation, structure elucidation and total synthesis of kirkamide, which was shown to be toxic to insects and arthropods.

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